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Uterine serpin (SERPINA 14) correlates negatively with cytokine production at the foetal-maternal interface but not in the corpus luteum in pregnant dairy heifers experimentally infected with Neospora caninum

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Complete List of Authors:	Serrano, Beatriz; University of Lleida, Animal Production Almeria, Sonia; Autonomous University of Barcelona, Mur-Novales, Ramón; University of Lleida, Animal Production López-Helguera, Irene; UdL, García-Ispierto, Irina; University of Lleida, Animal Production; ALABART, JOSE LUIS; Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón, Tecnología en Producción Animal Darwich, Laila; Autonomous University of Barcelona, Animal health and anatomy López-Gatius, Fernando; University of Lleida,
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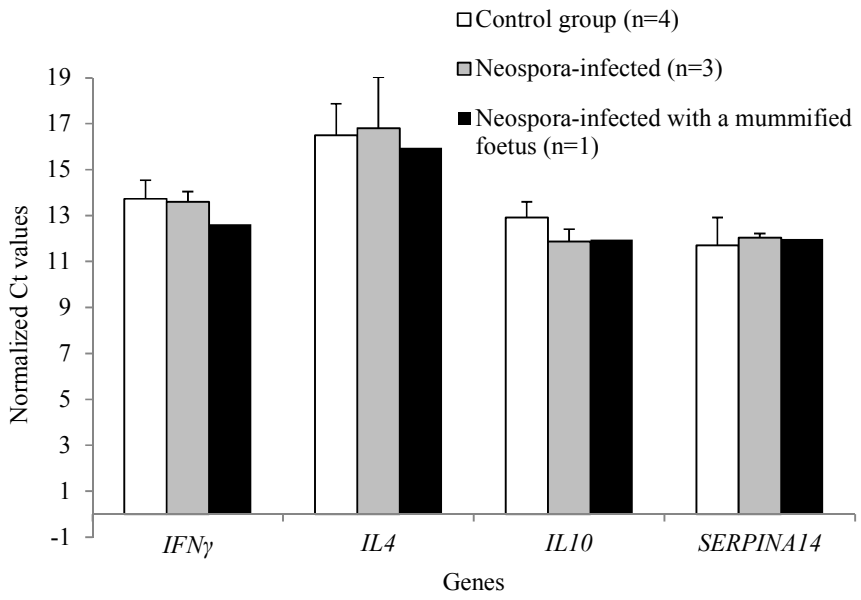
Figure 1



Uterine serpin (SERPINA 14) correlates negatively with cytokine production at the foetal-maternal interface but not in the corpus luteum in pregnant dairy heifers experimentally infected with *Neospora caninum*

Running title: Serpins and cytokines in *Neospora*-infected heifers

B. Serrano-Pérez[1,2], S. Almería[3,4], R. Mur-Novales[1], I. López-Helguera[1,2], I. Garcia-Ispierto[1,2], J.L. Alabart[5], L. Darwich[3,4], F. López-Gatius[2,6*]

[1]Department of Animal Science, University of Lleida, Spain.

[2]Agrotecnio Centre, University of Lleida, Spain.

[3]Centre de Recerca en Sanitat Animal (CReSA) - Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Campus UAB, 08193 Bellaterra, Barcelona, Spain

[4]Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Barcelona, Spain

[5]Instituto Agroalimentario de Aragón - IA2 - (CITA-Universidad de Zaragoza), Zaragoza, Spain

[6]Transfer in Bovine Reproduction SLu, 22300 Barbastro, Spain

*Correspondence, F. López-Gatius. Email address: lopezgatiusf@gmail.com

Contents

Neospora caninum is a major cause of abortion in cattle worldwide. However, immune-endocrine interactions during pregnancy in *Neospora*-infected cows remain largely unknown. This study examines gene expression patterns in dairy heifers experimentally infected with *N. caninum* during the second trimester of pregnancy that did not abort (n: 4 non-infected control dams and 4 infected dams). Based on the patterns observed, relationships were determined between gamma interferon (*IFNγ*), (Th1 pro-inflammatory cytokine), interleukin 4 (*IL4*) (Th2 pro-gestation cytokine) or interleukin 10 (*IL10*) (T regulatory cytokine) and the serine peptidase inhibitor *SERPINA14* in intercaruncular, placental, uterine lymph node (UTLN) and luteal tissue samples. Intercaruncular *SERPINA14* expression was negatively correlated with *IFNγ* expression in cotyledon samples (sr: -0.943; P= 0.005) and with *IL4* expression in UTLN (sr: -0.886; P= 0.019). A trend towards significance was also observed between intercaruncular *SERPINA14* expression and caruncular *IFNγ* expression (sr: -0.771; P= 0.072). Luteal tissue *IL10* expression was positively correlated with *IFNγ* and *IL4* expression both in luteal tissue (sr: 0.82; P=0.023 and sr: 0.714; P=0.071, respectively) and in caruncle samples (sr: 0.83; P=0.042 and sr: 0.88; P=0.019, respectively). No relationships were detected between cytokine gene expression at the foetal-maternal interface and *SERPINA14* expression in the luteal samples. Our findings indicate that, in these experimentally infected dams, gene expression of the uterine serpin *SERPINA14* correlates negatively with the expression of Th1 and Th2 cytokines at the foetal-maternal interface but not in the corpus luteum.

Keywords: Bovine neosporosis, pregnancy, maternal immune response, placenta, mummified foetus

Introduction

The mammalian foetus is an antigenically foreign body whose survival is the result of suppressed immunological interactions between mother and foetus (Druckmann 2001; Bauersachs and Wolf 2013). The key pregnancy hormone, progesterone, promotes mechanisms that induce this immunological tolerance of the foetus (Hansen et al. 1986). The immune response during pregnancy is mostly regulated through cytokines which are produced by T helper (Th) and T regulatory (Treg) cells. Cytokines are generally classified as "inflammatory cytokines" derived from Th1 cells and "pro-gestation cytokines" derived from Th2 cells. These two cytokine types stimulate cell-mediated immunity and promote the humoral response respectively (Mellor and Munn 2000). During the pregnancy period, progesterone seems to induce some Th2 bias, whereas an excessive Th1 response can induce pregnancy loss (Ragupathy 1997; Roberston 2000). Currently, it is not fully understood how the conceptus is able to avoid maternal immune attack. In addition, we would expect different conflict between reproductive and immune systems in healthy cows and those with a chronic infection.

Neospora caninum is an obligate, intracellular, protozoan parasite considered a major cause of abortion in cattle worldwide (Dubey and Schares 2011; Almería and López-Gatius 2015). On dairy farms, the major route of *N. caninum* infection is transplacental transmission from naturally infected dams to their foetuses during pregnancy (Goodswen et al. 2013; Almería and López-Gatius 2013). Most calves born to infected dams are clinically normal yet up to 95% of them remain infected for life (Dubey et al.

2007; Reichel et al. 2014). Abortion or congenital infection occurs when parasites cross the placenta and infect the foetus (Dubey and Lindsay 1996), and most abortions occur at 5-7 months of gestation (Almería and López-Gatius 2013). In our geographical area of study, northeastern Spain, *Neospora*-seropositive cows show a 12–19 times greater risk of abortion than seronegative cows, and the abortion rate ranges from 30 to 44% of seropositive animals (López-Gatius et al. 2004a,b). Foetuses may also die in utero and become mummified (Dubey and Lindsay 1996). The reasons why some animals abort and others do not remain unknown.

Th1 cytokines such as gamma interferon (IFN-γ) play an essential role in providing protective immunity against *N. caninum*. IFN-γ is a pro-inflammatory cytokine that inhibits the intracellular multiplication of *N. caninum* tachyzoites. However, an intense pro-inflammatory response, effective against *N. caninum* in non-pregnant cows, will be likely followed by foetal or placental damage and abortion in pregnant cows (Innes et al. 2005; Almeria and López-Gatius 2015). In cattle, IFN-γ has been linked to protection against *N. caninum*-associated abortion in several studies (Lopez-Gatius et al. 2007b; Williams et al. 2007; Almeria et al. 2012). Probably, the Th2 cytokine bias promotes maintenance of pregnancy by reducing local inflammatory responses (Wegmann et al. 1993; Chaouat et al. 2002). Our observation of the upregulated cytokine gene expression of both *IFNγ* and *IL4* (Th2) in infected dams reinforces this idea (Almería et al. 2016b).

It is known that *Neospora caninum* infection modifies endocrine patterns throughout gestation in dairy cattle. For example, *Neospora*-seropositivity has been associated with increased plasma prolactin and progesterone concentrations (Garcia-Ispierto et al. 2009,

2010) and reduced plasma concentrations of pregnancy-associated glycoproteins (PAGs) (Garcia-Ispierto et al. 2015). Pregnancy-associated glycoproteins I (PAG-I) and II (PAG-II) coexist in the ruminant trophectoderm (Zoli et al. 1991; Garbayo et al. 2008). Although the functions of PAGs are not yet fully understood, PAG-I and PAG-II concentrations in aborting animals are useful indicators of foetal-placental impairment (López-Gatius et al. 2007a; Garcia-Ispierto et al. 2013, 2105). In a recent study, expression patterns of the genes *SERPINA14*, *PAG1*, and *PAG2* at the foetal-maternal interface were investigated in dairy heifers experimentally infected with *N. caninum* during the second trimester of pregnancy (Serrano-Pérez et al. 2016). In infected dams with aborted foetuses, *SERPINA14* expression was significantly reduced and a negative relationship was observed between *N. caninum* antibody titres and *SERPINA14* or *PAG* expression in all infected animals (Serrano-Pérez et al. 2016).

The immunosuppressive actions of progesterone on the uterus during gestation have been attributed in part to uterine serpins (Hansen et al. 1987; Leslie and Hansen 1991). Serpins are glycoproteins and members of the serpin superfamily of serine peptidase inhibitors. One such serpin, SERPINA 14 (serpin peptidase inhibitor, clade A member 14), is expressed in response to progesterone in the endometrium (Ing and Roberts 1989) and has been linked to maternal immunosuppression during pregnancy (Padua and Hansen 2010). In pregnant ruminants, besides its presence in the endometrium, SERPINA14 also occurs in ovarian luteal and follicular structures (Ulbrich et al. 2009). Since *Neospora*-infection seems to reduce *SERPINA14* gene expression (Serrano-Pérez et al. 2016) and increase *IFNγ* and *IL4* gene expression (Almería et al. 2016b), we hypothesized that *SERPINA14* gene expression would be negatively correlated with the expression of *IFNγ* and *IL4* genes in pregnant dams suffering *Neospora*-infection.

The present study is one of a series of investigations performed during the second trimester of gestation in pregnant dairy heifers experimentally infected with *N. caninum* on day 110 of gestation. This stage of pregnancy was selected as the time when most abortions occur in field conditions. The objective of this study was to detect possible correlation between expression of the genes *IFNγ*, *IL4,* interleukin 10 (Treg) (*IL10*) and *SERPINA14* at the foetal-maternal interface on day 152 of gestation in *Neospora*-infected non-aborting animals. Since tropism of *N. caninum* for the ovarian follicle has been suggested (Silva et al. 2012), a second objective was examine the same gene expression patterns in the corpus luteum and their possible relationships with *Neospora*-infection and with cytokine gene expression at the foetal-maternal interface.

Material and methods

Animals and infection

The animals used and the infection protocol have been described elsewhere (Almeria et al. 2016a). Briefly, ten 14-16 month-old Holstein-Friesian heifers free of abortifacient agents and seronegative for *N. caninum* (CIVTEST® anti-*Neospora*; Hipra, Girona, Spain) were synchronized for oestrus and artificially inseminated. Seronegativity was confirmed before insemination and on Days 60, 90 and 110 of gestation. Pregnancy was assessed by ultrasonography 30, 45, 90 and 110 days after insemination. On Day 110 of gestation, six of the heifers were intravenously (i.v.) inoculated with 10^7 culture-derived tachyzoites of the *N. caninum* isolate Nc-Spain7, kindly donated by Dr. L. M. Ortega-Mora (SALUVET, Universidad Complutense, Madrid, Spain). Two heifers that aborted

at 14 and 21 days post-infection were excluded from the study. Another heifer had a mummified foetus upon euthanasia. The four non-aborting animals were euthanized on Day 152 of gestation. The four remaining heifers were kept as un-inoculated controls and were euthanized at the same time as the inoculated dams.

Sample collection

On Day 152 of gestation (Day 42 post-infection), blood samples were collected by tail vein puncture into heparinized vacuum tubes (BD Vacutainer, Becton-Dickinson and Company, Plymouth, UK) to determine maternal antibodies and progesterone concentrations. Plasma obtained by centrifugation within 30 min of sampling was stored at -20ºC until analysis. After blood collection, all animals were sedated with xylazine hydrochloride (Rompun; Bayer, Sant Joan Despí, Barcelona, Spain) and immediately euthanized by an intravenous (i.v.) overdose of embutramide and mebezonio iodide (T61; Intervet, Salamanca, Spain). Immediately after sacrifice, heifers were necropsied and tissues were removed aseptically according to Almería et al. (2016a). The uterus was removed and foetal amniotic and allantoic fluids collected by puncture (Mur-Novales et al. 2016). Next, the uterus was opened and foetal tissue and blood samples were collected. Portions of foetal tissues were aseptically obtained and stored in liquid nitrogen at -196ºC until DNA extraction. Tissues collected from foetuses were: CNS (brain and spinal cord), heart, lung, liver, skeletal muscle, spleen, and thymus. The lymphatic vessels draining the uterus, or internal iliac lymph nodes referred to here as uterine lymph nodes (UTLN), were collected from the heifers for the isolation of mononuclear cells as described by Almeria et al. (2014). In addition, samples of three selected placentomes (cranial, medial, and caudal placenta) were removed from each

dam. Both the maternal side (caruncle) and foetal side of the placenta (cotyledon) were careful separated manually from each placentome. Intercaruncular tissue was also collected. The corpora lutea were aseptically dissected and divided into two sections: one was stored in liquid nitrogen and the other section was used for histopathology.

Ethics

All procedures were approved by the Ethics Committees on Animal Experimentation of the Autonomous University of Barcelona (license number CEEAH.1426-08/02/2012) and University of Lleida (license number CEEA.06-01/12). Animals were handled in strict accordance with good animal practices and the conditions defined by the Animal Ethics Committee of the Autonomous University of Barcelona and CReSA, Spain. Every effort was made to minimize suffering.

Sample analysis

Progesterone assay and corpus luteum histopathology

Progesterone concentrations were determined in plasma samples using an ELISA kit designed for bovine plasma (Ridgeway Science, St. Briavels, Gloucestershire, UK), according to the manufacturer's instructions. Assay sensitivity was 0.33 ng/mL. All samples were analysed in duplicate in the same assay. Intra-assay coefficients of variation for sample pools of 1, and 2 ng/mL were 6.4 and 4.8%, respectively.

Paraffin-embedded 5-µm sections of corpora lutea were prepared and stained with haematoxylin-eosin for histopathological examinations.

Total RNA extraction and cDNA synthesis

Intercaruncular, UTLN, placental and luteal tissue samples were kept frozen in liquid nitrogen, homogenized in a mortar in the presence of additional liquid nitrogen and maintained in trizol (Invitrogen Corp., Carlsbad, CA, USA) at -80ºC. For caruncle or cotyledon tissue gene expression analysis, a mixed sample of RNA from the three different sections (cranial, medial and caudal) of each tissue was used as template.

Total RNA was extracted according to the method of Chomczynski and Sacchi (1987). Samples were treated with DNAse in the presence of RNAse inhibitors to eliminate contaminating genomic DNA. Concentrations of RNA were determined spectrophotometrically. RNA integrity was checked by denaturing agarose gel electrophoresis. Complementary DNA was synthesized from 2 µg of total RNA in the presence of random primers using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations.

Real time RT-PCR

Messenger RNA expression was determined by real time RT-PCR on four target genes: *IFNγ*, *IL4, IL10* and *SERPINA14*.

Cytokine gene expression

Cytokine gene expression (*IFNγ*, *IL4, IL10*) was determined in UTLN, placental and luteal samples. Messenger RNA expression was determined by real time RT-PCR following the Taqman approach in an ABI PRISM™ 7700 sequence detector (PE Applied Biosystem, Foster city, CA, USA). Probes and primers for bovine *IL4* and *IFNγ* were those reported by Waldvogel et al. (2000). The bovine *IL10* sequence probes and primers used have been described in Almeria et al. (2003). Probes and primer pairs were used to quantify *GAPDH* RNA as the endogenous housekeeping control gene described by Leutenegger et al. (2000). Primer and probe concentrations for each cytokine were determined as previously reported (Almeria et al. 2003, 2011). PCR amplifications were conducted as in Almeria et al. (2014). Endogenous *GAPDH* housekeeping expression was used to normalize levels of cytokine gene expression.

SERPINA14 gene expression

SERPINA14 gene expression was determined by real-time PCR in intercaruncular, placental and luteal samples. *SERPINA14* mRNA expression was determined using the SYBRgreen method and ABI PRISM™ 7500 sequence detector (Applied Biosystem, Foster City, CA, USA). The gene β-actin (*ACTB*) was used as housekeeping gene to normalize levels of *SERPINA14* gene expression. The primers used for *ACTB* and *SERPINA14* have been described elsewhere (Ribeiro et al. 2014; Serrano-Pérez et al. 2016). Amplifications were performed as described in Serrano-Pérez et al. (2016).

For relative quantification of gene expression, the comparative threshold cycle (CT) method (ABI PRISM7700 sequence detection system, user bulletin #2) was used as described in Almeria et al. (2003). Briefly, the CT value for the housekeeping gene was subtracted from the CT value for each target expression gene to normalize RNA content and provide a relative expression value for each target gene. This value was defined as ΔCT. To assess the effects of infection, the mean ΔCT for the control dams was subtracted from the mean ΔCT determined for the *Neospora*-infected dams. This value was defined as ΔΔCT. Relative fold increases or decreases were then calculated as $2^{-\Delta\Delta CT}$.

Statistical analysis

Spearman's rho (sr) test was used to identify possible relationships between gene expression levels of each cytokine and *SERPINA14* in uninfected controls and infected animals. The Student's t-test was used to compare relative *SERPINA14* and cytokine mRNA expression in luteal samples and progesterone concentrations among uninfected controls and infected dams. Only dams with viable foetus upon euthanasia were included in the latter statistical analysis. All tests were performed using the computer package SPSS version 17.0 (SPSS Inc., Chicago, IL). Significance was set at $P \leq 0.05$.

Results

The present study was designed on the basis of the results described in Almería et al. (2016). In this prior work, all experimentally infected heifers were seropositive for *N. caninum* upon euthanasia, and transplacental infection had already taken place in their

foetuses. Control uninfected foetuses showed no antibodies and *N. caninum* DNA was not detected in any of their tissues. The mummified foetus was estimated to have died approximately 28 days after infection (Almería et al. 2016a). Due to the lack or poor condition of some samples, a variable number of samples for cytokine and *SERPINA14* gene expression were available: samples from seven animals for UTLN and caruncle tissues (controls, n=3; infected dams, n=4), samples from eight animals for intercaruncular and luteal tissues (controls, n=4; infected dams=4), and samples from six animals for cotyledon tissues (controls, n=3; infected dams=3).

Gene expression levels in the dam with the mummified foetus were similar to those in the remaining three infected dams in the intercaruncular (*SERPINA14*), UTLN (*IFNγ*, *IL4, IL10*) and luteal tissues (*IFNγ*, *IL4, IL10, SERPINA14*).

Plasma progesterone determinations and luteal histopathology

Neospora-infection did not affect plasma progesterone concentrations (mean ± S.D. values: 12.53 ± 1.5 ng/ml in controls vs. 12.85 ± 3.7 ng/ml in infected dams) and microscopic lesions were not observed in the luteal samples of any of the 4 infected dams.

Correlations between *SERPINA14* and cytokine gene expression in UTLN, intercaruncular and placental tissues

SERPINA14 expression in intercaruncular samples was negatively correlated with *IFNγ*, expression in cotyledon samples (sr: -0.943; P= 0.005) and with *IL4* expression in

UTLN samples (sr: -0.886; P= 0.019). Trends towards significance were also observed between *SERPINA14* expression in intercaruncular samples and *IFNγ* in caruncle samples (sr: -0.771; P= 0.072). No other significant correlations were found.

Gene expression of *SERPINA14* and cytokines in luteal samples

Luteal samples from uninfected heifers did not reach detection threshold levels of expression for *IL4*. When normalized levels were compared, no relationships were detected between any cytokine and *SERPINA14* gene expression. In addition, no significant differences were detected between the gene expression of cytokines and *SERPINA14* in luteal samples from infected dams and control dams (Figure 1). Cytokine and *SERPINA 14* gene expression levels detected in the infected dam with a mummified foetus upon euthanasia, not included in our statistical analysis, are also shown in Figure 1.

Correlating *SERPINA14* and cytokine gene expression between luteal and foetal-maternal interface samples

The expression of *IL10* in luteal samples was positively correlated with *IFNγ* and *IL4* expression in both luteal samples (sr: 0.82; P=0.023 and sr: 0.714; P=0.071, respectively) and caruncle samples (sr: 0.83; P=0.042 and sr: 0.88; P=0.019, respectively). No relationships were detected between any cytokine expressed at the foetal-maternal interface and *SERPINA14* gene expression in the luteal samples.

Discussion

This study unveils several aspects of immune-reproductive modulation at the level of the foetal-maternal interface in response to *N. caninum* infection during the second trimester of gestation in dairy heifers. Relationships between patterns of gene expression shown by a uterine serpin (*SERPINA14*) and by the cytokines Th1 (*IFNγ*), Th2 (*IL4*) and Treg (*IL10*) in UTLN, intercaruncular, placental and luteal tissues provided useful insight into this modulation. The most noteworthy findings of the present study were that *SERPINA14* gene expression in intercaruncular tissues correlated negatively with *IFNγ* gene expression in the cotyledons and with *IL4* in the UTLN tissue samples, whilst *IL10* expression in corpus luteum correlated positively with *IFNγ* and *IL4* in both luteal and placental tissues.

Immunosuppressive function downregulation by SERPINA 14 in response to *N. caninum* infection was suggested in a prior study (Serrano-Pérez et al. 2016). In effect, the negative correlation observed here between *SERPINA14* expression and Th1 and Th2 cytokines likely indicates that reduced local immunosuppression by the uterine serpin is needed to improve maternal immune responses against the parasite to maintain gestation. These data support recent findings involving the production *in vitro* of the cytokines IFN-γ (Th1) and IL-4 (Th2) whereby a protective immune response against abortion could not be associated with IFN-γ levels alone, but was significantly linked to lower IFN-γ/IL-4 ratios (Darwich et al. 2016). Thus, the Th1/Th2 balance seems to play a key role in maintaining pregnancy during the course of *N. caninum* infection.

Our observation of significant positive correlation between the luteal expression of Treg (*IL10*) and the expression of Th1 (*IFNγ)* and Th2 (*IL4)* in both luteal and placental

tissues is consistent with several reports of simultaneously up-regulated expression of the different cytokine subsets in pregnant cattle at the placental level during *N. caninum* infection (Rosbottom et al., 2008; Almeria et al. 2011, 2014, 2016b; Cantón et al. 2014; Regidor-Cerrillo et al., 2014; Hecker et al. 2015). Thus, it seems the placenta can act as an immune organ that can sense changes in the environment and then signal to other cells to elicit a response (Schminkey and Groer 2014; PrabhuDas et al. 2015). The currently held type 1/type 2 paradigm of pregnancy indicates that to maintain uterine quiescence, Th2 cytokines predominate throughout mid-pregnancy, while the beginning and end of pregnancy are times of type 1 predominance allowing for implantation and parturition. This mechanism could be controlled by the innate immune system, which responds to the pregnancy in much the same way as it responds to other acute or naturalistic stressors (Schminkey and Groer, 2014).

While *SERPINA14* was observed to correlate negatively with cytokine production at the foetal-maternal interface, no relationships were detected between any cytokine and *SERPINA14* gene expression in luteal samples. In addition, despite suggestions of possible tropism of *N. caninum* for ovarian follicles (Silva et al. 2012), in our experimental conditions, *N. caninum* infection had no clear effects on the immune response taking place in the corpus luteum. Therefore, it seems that the infection had no impacts on corpus luteal function, at least in terms of repercussions on plasma progesterone concentrations and expression of the genes coding for *SERPINA14* and the cytokines *IFNγ*, *IL4* and *IL10*.

In conclusion, our findings indicate that the gene expression of the uterine serpin *SERPINA14* correlates negatively with the expression of Th1 and Th2 cytokines at the

foetal-maternal interface but not the corpus luteum in dams experimentally infected with *N. caninum* during the second trimester of gestation. These data are consistent with our starting hypothesis that expression of the *SERPINA14* gene would correlate negatively with the expression of the *IFNγ* and *IL4* genes.

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Figure 1. Relative expression levels of the genes *SERPINA14*, *IFNγ*, *IL4* and *IL10* in luteal samples from control dams (n=4), *Neospora*-infected dams with viable foetuses (n=3), and dams with a mummified foetus (n=1) on Day 152 of gestation upon euthanasia. The higher the normalized CT value the lower the expression level. Bars represent mean Ct values ± standard error of the mean.